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NATURE AND LOCALIZATION OF ACIDIC GROUPS ON LYSOSOMAL MEMBRANES

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SUMMARY

pH-dependent reversible binding of lysosomal enzymes to membranes of Triton WR 1339-filled rat liver lysosomes indicates the presence of acidic groups on lysosomal membranes. Increasing the ionic strength as well as addition of nonlysosomal proteins inhibit binding of enzymes at pH 4.

Digestion of lysosomal membranes with proteases and phospholipase C has no effect on the reversibility of enzyme–membrane binding. Only neuraminidase treatment of lysosomal membranes significantly reduces the enzyme–membrane binding at pH 4 indicating that sialic acid is the main anionic group at this pH.

Electron micrographs of colloidal iron-stained lysosomes show a preferential localization of sialic acid on the inside of the lysosomal membrane. Neuraminidase-treated lysosomal membranes show no or considerably reduced iron staining.

pH-dependent binding curves of lysosomal enzymes to a strong acid cation-exchanger (phosphocellulose) show a similar shape as those obtained with lysosomal membranes. The intralysosomal accumulation of nondiffusible anions might produce a Donnan equilibrium serving as an energy independent means to maintain an intralysosomal acid milieu.

INTRODUCTION

The lysosomal membrane plays a key role in the lysosome concept as worked out by De Duve *et al*[1]. Rupture of this membrane renders lysosomes permeable to substrates and allows enzymes to leak out. Injuries can be caused by mechanical or osmotic means, repeated freezing-thawing, surface active detergents, thermal activation and by low pH. These findings have led to the suggestion that lysosomal enzymes are, at least in part, freely diffusible within lysosomes. Furthermore, the lysosome concept implies that intracellular digestion of pinocytized or autophagocytized material occurs within lysosomes requiring the full catalytic activity of the hydrolases within lysosomes. The lysosomal membrane, therefore, protects intracellular compounds and structures from the attack of lysosomal enzymes. Since the lysosomal membrane is rather sensitive even within the living cell against reversible cell injuries, additional modes of protecting the cell from the attack of lysosomal enzymes are

required. This is achieved by the acid pH optimum of most of the lysosomal enzymes being far out of the range of normal intracellular pH. In consequence, the full intralysosomal activity of hydrolases requires an acid milieu within lysosomes.

The pH within lysosomes has been postulated to be acid (de Duve[2]). Besides some rough estimations by the use of indicator dyes[3,4,29] the intralysosomal pH has been determined recently by Reijngoud and Tager[5]. These authors found a pH up to 1.5 units lower than in the surrounding medium.

Among the mechanisms maintaining an intralysosomal acid pH Coffey and de Duve[6] discussed the possibility of an intralysosomal accumulation of nondiffusible anions setting up a Donnan potential. Support for this hypothesis may be found in the description of a strongly acidic component in lysosomes of kidney by Dingle and Barrett[7] as well as a report on the occurrence of acidic lipoproteins by Goldstone *et al.*[8] in lysosomes of rat kidney and liver. Mego and associates[9] have postulated an ATP-dependent proton pump in the lysosomal membrane.

Previous studies in this laboratory on the formation of secondary lysosomes induced by the nonionic detergent Triton WR 1339 demonstrated the pinocytotic uptake of this compound[10,11]. This method, introduced by Wattiaux *et al.*[12], allows the isolation of a pure fraction of secondary lysosomes meeting all criteria required for the characterization of lysosomes with its complete spectrum of enzymes and the latency of the enzymatic activity.

The purity of this lysosome fraction prompted us to show the presence of anionic charges bound to the insoluble fraction, to describe their localization and their characterization in terms of the chemical structure.

METHODS

Triton WR 1339 filled lysosomes were prepared from livers from Triton WR 1339 injected rats (96 h after injection, 170 mg/100 g rat) by slight modifications[13] of the method of Wattiaux[12]. As shown by the determination of marker enzymes[13] and cytochromes[32] as well as by electron microscopy tritosomes were free of contaminating organelles. Electron micrographs show no detectable contamination with mitochondria, endoplasmic reticulum and peroxisomes[11,32]. Rat liver plasma membranes and light mitochondrial fractions (750–12000 × *g* for 10 min sediments) were prepared and examined according to previously described methods[13,14]. Protein was determined by the method of Lowry *et al.*[15]. Tritium labelled Triton WR 1339 was kindly prepared by Prof. Dr G. Stöcklin, Kernforschungsanlage Jülich, Germany, using the Wilzbach technique.

The integrity of the lysosomal membrane and the binding of lysosomal enzymes (acid phosphatase and β-glucuronidase) to the sedimentable fraction were determined by the following three assays:

(1) Tritosomes prepared from rats injected with [^{3}H]Triton WR 1339 (total activity injected: $5 \cdot 10^7$ dpm/100 g rat) were incubated under conditions indicated in the figures and spun down at 20000 × *g* (15 min). The released [^{3}H]Triton was measured in the supernatant and given in percent of total radioactivity. Previous results[10,11] indicated that the component of Triton WR 1339 taken up by pinocytosis has a molecular weight of over 100000 and a diameter of 85 Å (determined by electron

microscopy; see Discussion). Therefore, the [^{3}H]Triton WR 1339 release allows a judgement on the integrity of the lysosomal membrane.

(2) Determination of "free" enzyme activity according to Appelmans and de Duve[16]: After incubation of lysosomal preparations under conditions indicated in the legends of the figures "free" activity was measured in the presence of an osmotically protected buffer substrate mixture (0.25 M sucrose) by short term incubations to avoid thermal activation. Under these conditions the granules remain mostly intact and the enzymes enclosed in lysosomes are inactive. This assay covers both freely dissolved enzymes and enzymes bound to injured lysosomes (ghosts) and therefore allows the estimation of the integrity of the membrane.

(3) Determination of the nonsedimentable enzyme activity: After preincubation lysosomes or phosphocellulose (used for model experiments to study the binding behaviour of β-glucuronidase to a strong cation-exchanger) were spun down routinely at 20000 $\times$ g (15 min) and the enzyme activity was determined in the supernatant. No change of enzyme activity and radioactivity was found in the supernatant after high speed centrifugation (105000 $\times$ g, 60 min). This procedure assays the enzyme binding to the insoluble fraction of the lysosomes.

Total enzyme activities (= 100%) were determined in the presence of 0.1% Triton X 100. Total radioactivity was measured from 0.1 ml aliquots after the addition of 0.5 ml of Soluene (Packard) in a toluene scintillator fluid. Acid phosphatase was assayed according to Linhardt and Walter[17] using *p*-nitrophenyl phosphate as substrate and β-glucuronidase according to Gianetto and De Duve[18] using *p*-nitrophenyl-β-D-glucopyranosiduronic acid.

Enzymic digestion of lysosomal membranes (0.3 mg of protein per assay) was performed under the following conditions: Pronase (Serva, Heidelberg, Germany) 1 mg, 0.1 M Tris–HCl buffer, pH 7.6; papain (Serva) 1 mg, Tris–HCl buffer 0.1 M, pH 7.6, cysteine 5 mM, EDTA 1 mM; trypsin (Difco, Nordwald KG, Hamburg) 1 mg, 0.1 M Tris–HCl buffer, pH 8.1, 10 mM $CaCl_2$; phospholipase C (*Clostridium welchii*, Calbiochem, Paesel KG, Frankfurt/Main) 0.2 mg, 0.1 M Tris–HCl buffer, pH 7.2; neuraminidase 50 units (*Vibrio comma cholerae*, Behringwerke, Marburg, Germany), 0.1 M acetate–barbiturate buffer, pH 6.5, 5 mM $CaCl_2$. The incubations were carried out in a total volume of 1.0 ml for 3.5 h at 37 °C. Sialic acid was determined by the method of Warren[21].

Samples used for electron microscopy were light mitochondrial fractions from normal rat livers, Triton WR 1339 filled lysosomes prepared 10, 24 and 72 h after Triton injection and a "heavy" lysosomal fraction filled with Triton WR 1339 (density 1.12–1.16 g/ml contaminated with mitochondria) isolated 10 and 24 h after Triton injection as described earlier[14]. Fixation was performed with double-distilled glutaraldehyde (6.25% in 0.1 M cacodylate–HCl buffer, pH 7.2, 0.25 M sucrose). Staining with colloidal iron (pH 1.7) of fixed suspensions of organelles followed the method of Gasic *et al.*[19]. No osmium tetroxide was applied to these pellets because it might interfere with the staining reaction[19]; aliquots for normal morphology were studied with and without additional OsO_4 fixation. Samples were dehydrated with acetone and embedded in the araldite "Durcupan"^{R} ACM (Fluka, Buchs, Switzerland).

Ultrathin sections of silver interference colour from pellets stained with colloidal iron were investigated either uncontrasted or after contrasting for 3 h with 2% uranyl

acetate in ethanol at 60 °C (Locke and Krishnan[20]). In addition sections from morphological controls were alternatively stained also either with routine methods, *i.e.* 20 min with 7.5% aqueous magnesium uranyl acetate followed by 10 min alkaline lead citrate (Venable and Coggeshall[25]), or according to Ainsworth and Karnovsky[26].

The following controls were performed: (a) Digestion of unfixed aliquots with neuraminidase (100 units/ml, Behringwerke, Marburg, Germany) under conditions described above (pH 6.5) followed by glutaraldehyde fixation and exposure to the colloidal iron solution. (b) The occurrence and distribution of electron dense material in lysosomes without colloidal iron staining was carefully registered under all fixation and contrasting conditions used. Ferritin particles as a common constituent of the lysosomal matrix were identified by comparing their endogenous electron scattering after glutaraldehyde fixation without section staining and after section staining with alkaline bismuth tartrate (Ainsworth and Karnovsky[26]).

All micrographs were taken from unsupported sections in a Siemens Elmiskop I electron microscope under identical operating conditions using 80 kV accelerating voltage, a 50 μm selfcleaning "French" objective aperture and an anticontamination device cooled with liquid nitrogen.

RESULTS

Free and nonsedimentable activities of acid phosphatase and β-glucuronidase have been determined after preincubation at various pH values of a light mitochondrial fraction (crude, lysosome rich fraction) of normal rat liver. The results shown in Fig. 1 confirm well the early observations of Appelmans and de Duve[16] in that the lysosomal membrane is pH sensitive and that after injury of the membrane the enzymes are released into the medium. In the case of acid phosphatase the difference between free and nonsedimentable activities is more distinct suggesting a stronger but pH independent binding to sedimentable structures.

Incubation of pure Triton-filled lysosomes leads to opposing results with regard to the nonsedimentable activity. As Fig. 2 shows release of [^{3}H]Triton WR 1339 and the free enzyme activities increase with acidification, demonstrating again the pH sensitivity of the membrane whereas the nonsedimentable activities decrease with acidification.

This pH-dependent binding to lysosomal "ghosts" is due to ionic forces. The isoelectric points of acid phosphatase (pH 4.5[22]) and β-glucuronidase (pH 5.8[23]) indicate that both enzymes are positively charged at pH 4. The lysosomal membrane as binding counterpart should therefore be negatively charged at this pH.

The ionic nature of this binding of β-glucuronidase to lysosomal ghosts can be demonstrated by increasing the ionic strength at pH 4. Fig. 3 shows the dependence of binding of this enzyme to lysosomal membranes on the ionic strength increased by various salts at pH 4.

Addition of nonlysosomal proteins such as albumin or even other rat liver subcellular material inhibits binding of β-glucuronidase at pH 4 as seen in Fig. 4. The albumin was dialyzed extensively to exclude the influences of ions. Mitochondria were used to show the influence of contamination of lysosomal fractions with other subcellular elements. This experiment demonstrates that data reported hitherto on the

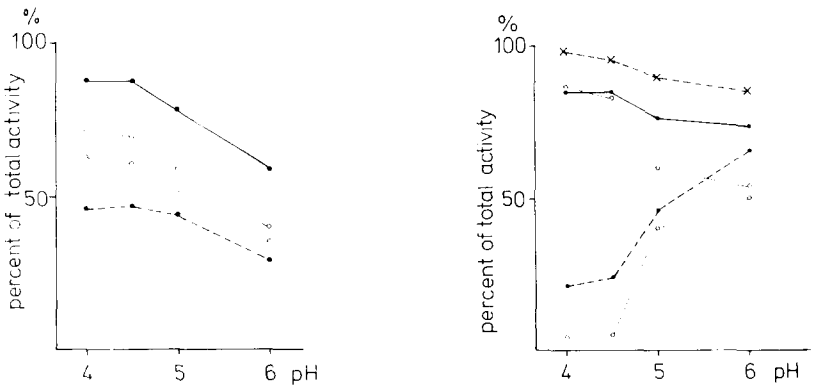


Fig. 1. Free and nonsedimentable activities of acid phosphatase and β-glucuronidase of a lysosome rich fraction of normal rat liver (light mitochondrial fraction). Enzyme activities were determined as outlined under Methods after preincubation of 0.3 mg of protein in 1.0 ml 0.037 M acetate buffer of desired pH (0.25 M sucrose) for 30 min at 37 C. ●–●, acid phosphatase; – –, β-glucuronidase; · · ·, "free" activity; – – – –, nonsedimentable activity.

Fig. 2. Free and nonsedimentable activities of acid phosphatase and β-glucuronidase of Triton WR 1339 filled lysosomes, release of [^{3}H]Triton WR 1339. Enzyme activities were determined after preincubation of 0.3 mg of protein in 1.0 ml 0.037 M acetate buffer (0.25 M sucrose) of desired pH for 10 min at 37 C. ×–×, release of [^{3}H]Triton WR 1339 ●–● acid phosphatase; – · · –, β-glucuronidase; – · –, "free" activity; – · – –, nonsedimentable activity.

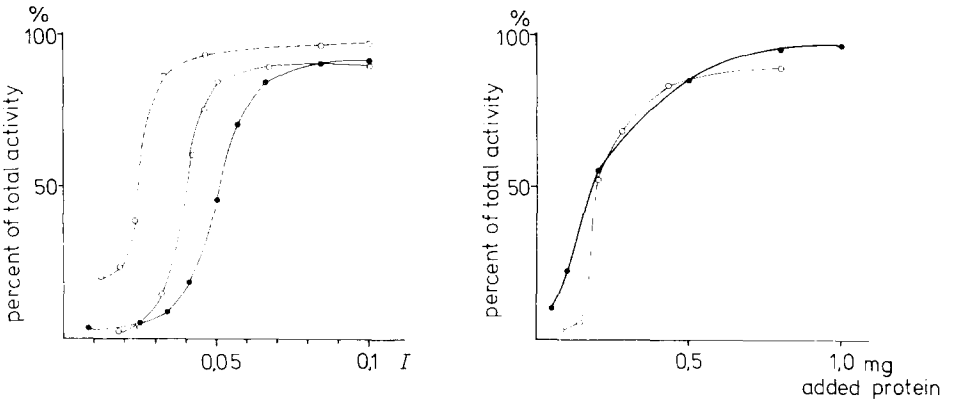


Fig. 3. Influence of ionic strength on the β-glucuronidase binding to Triton WR 1339 filled lysosomes at pH 4. Nonsedimentable activity was determined after incubation at pH 4 (0.037 M acetate buffer, 0.25 M sucrose, total vol. 1.0 ml) with increasing ionic strength (I). – – –, $MgCl_2$; · · ·, $MgSO_4$; ●——●, NaCl.

Fig. 4. Influence of nonlysosomal proteins on the β-glucuronidase binding to Triton WR 1339 filled lysosomes at pH 4. Nonsedimentable activity was determined after incubation at pH 4 (0.037 M acetate buffer, 0.25 M sucrose, total vol. 1.0 ml) with increasing amounts of nonlysosomal protein. ●——●, dialyzed bovine serum albumin; · · ·, rat liver mitochondria.

sedimentability of lysosomal enzymes in lysosome rich but not very pure fractions are questionable.

Binding and release of β-glucuronidase to lysosomal membranes is reversible. This allows the study of enzyme-binding to lysosomal membranes which have been treated with various enzymes in order to recognize the chemical nature of the anionically charged molecules. Lysosomal membranes were prepared by osmolysis of isolated Triton-filled lysosomes with subsequent centrifugation (105000 × *g*, 60 min). The membranes were separated from the supernatant containing the lysosomal enzymes and treated with exogenous hydrolytic enzymes as indicated under Methods (trypsin, papain, pronase, phospholipase C, neuraminidase). After the enzyme treatment, the insoluble fraction was spun down (20000 × *g*, 15 min) and washed twice to remove remnants of the enzyme as well as of the incubation medium. The washed lysosomal membrane fraction was mixed with the equivalent amount of the supernatant containing the original lysosomal enzymes and adjusted to pH 4 (acetate buffer, final concentration 0.012 M). After short term incubation (5 min, room temperature) membranes were spun down.

The following controls were performed. The influence of endogenous lysosomal enzymes possibly adherent to the membrane fraction was excluded by control incubations with lysosomal membranes which had been treated for 30 min in boiling water. No effect on the reversible pH dependent binding of β-glucuronidase to lysosomal membranes was found after this procedure. The effect seen after extensive neuraminidase treatment (Table I) was ascertained by the use of heat treated neuraminidase (30 min 100° C) which also had no effect on the reversible enzyme membrane binding (Table I).

The results shown in Table I indicate that sialic acid residues apparently are the only important anionically charged groups on lysosomal membranes at pH 4. This property of lysosomal membranes seems to be specific since no pH-dependent binding

TABLE I

REVERSIBILITY OF ENZYME BINDING TO LYSOSOMAL MEMBRANES AT pH 4 AFTER DIGESTION OF MEMBRANES WITH VARIOUS ENZYMES

Lysosomal membranes prepared (for details see text) and digested with enzymes indicated in the table (0.3 mg membrane protein per assay). After digestion, membranes were spun down, washed and incubated with the appropriate amount of lysosomal contents at pH 4 (0.012 M acetate buffer, 5 min, 22° C). After terminating the incubation by centrifugation the enzyme activity (β-glucuronidase) bound to lysosomal membranes was determined in % of total activity present per assay.

Lysosomal membranes digested with	*% of β-glucuronidase bound to membranes*
Neuraminidase	66
Heat inactivated neuraminidase	96
Trypsin	97
Papain	96
Pronase	97
Phospholipase C	97

of lysosomal enzymes by plasma membranes, mitochondria or microsomes could be observed. This point is stressed in more detail in the Discussion because of the importance of the plasma membrane bearing sialic acid on the outside[24] with regard to the inside occurrence of sialic acid in lysosomes.

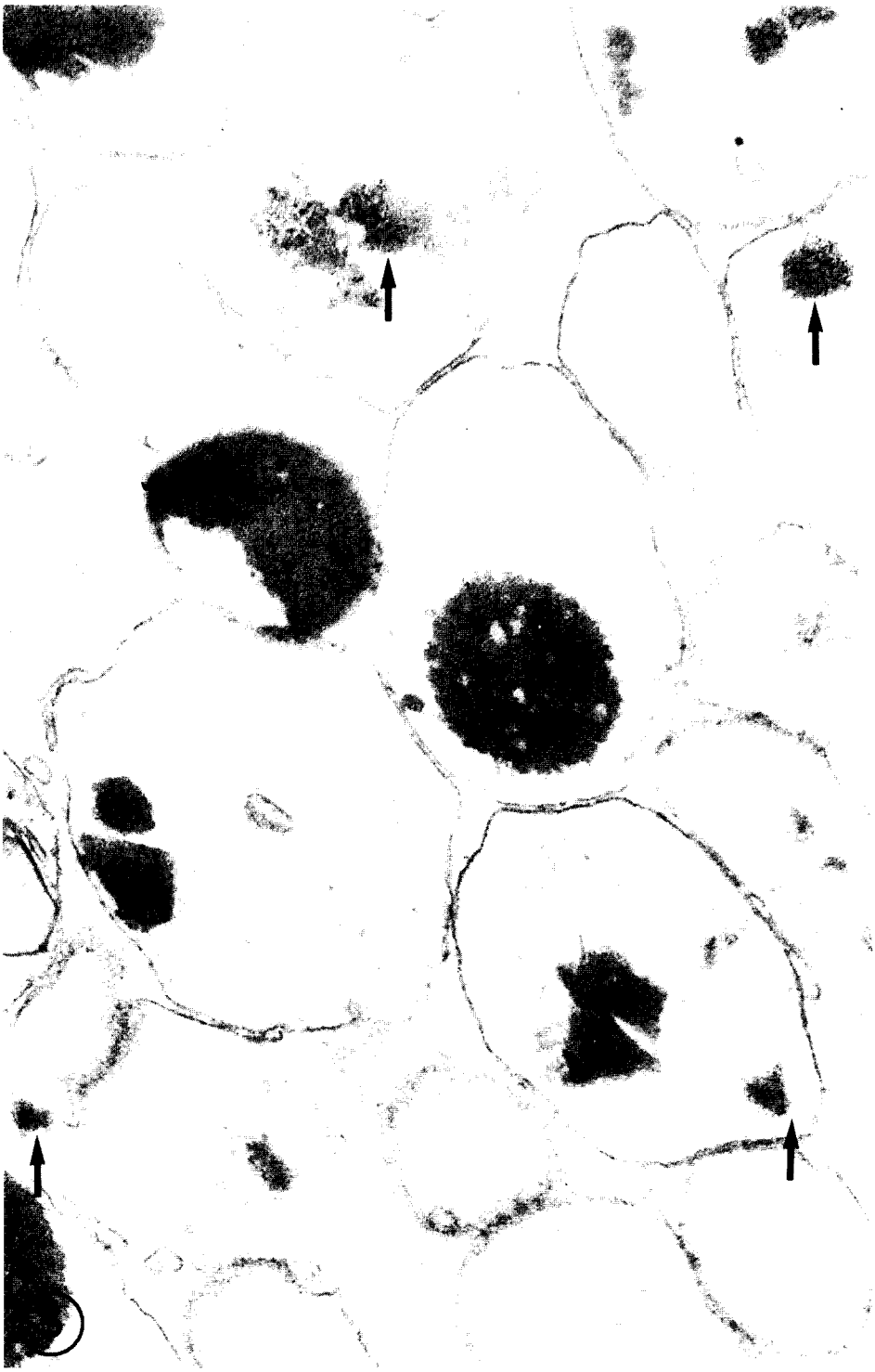
Attempts were undertaken to localize the sialic acid on the in- and/or outside of lysosomal membranes. Determinations of the sialic acid concentrations (a) of untreated lysosomal membranes (14.9 ± 1.9 μg/mg protein), (b) of lysosomal membranes prepared after neuraminidase treatment of lysosomes assumed to be mostly intact (digestion from outside) (9.8 ± 5.0 μg/mg protein) and (c) of lysosomal membranes (7.8 ± 1.1 μg/mg) treated with neuraminidase after disruption of lysosomes (digestion from both sides) gave no conclusive results. Injury of lysosomes during neuraminidase treatment in experiment (b) (digestion from outside) could not be avoided (as determined by the release of [^{3}H]Triton WR 1339) and amounted to approximately 40% even in short term experiments at room temperature (30 min, 22 °C).

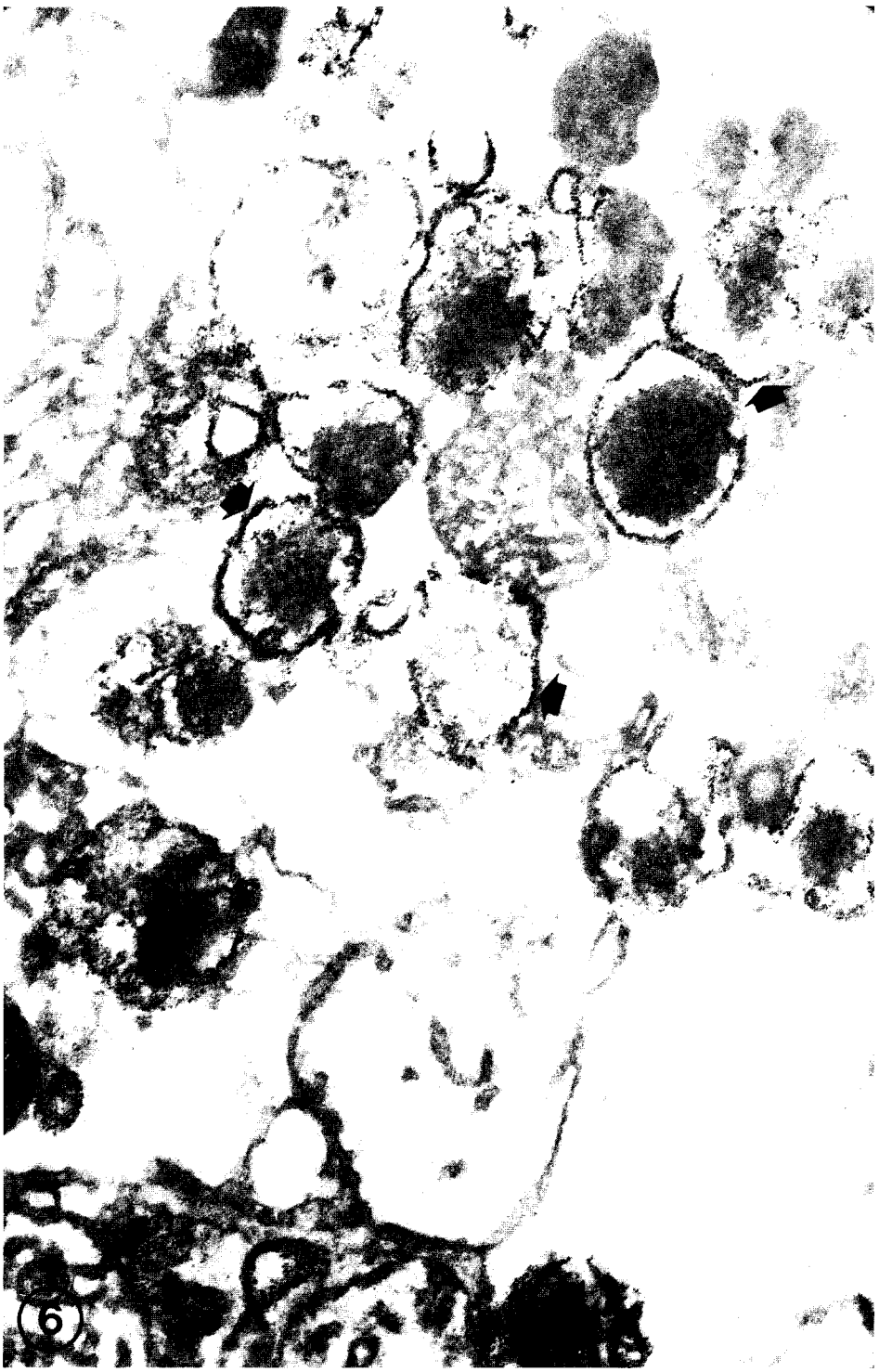
Unequivocal results were obtained by electron microscopy using the colloidal iron staining technique according to Gasic *et al.*[19]. Controls (Fig. 5) were analyzed in order to ascertain that the staining reaction does not interfere with endogenous constituents: 50 Å large particles with endogenous electron scattering are mostly associated with some amorphous matrix material of moderate electron density after standard fixation and section contrasting techniques. These particles are considered to represent ferritin (Ainsworth and Karnovsky[26]) which is frequently encountered in rat liver lysosomes (Novikoff[27], Wattiaux *et al.*[12], Baudhuin *et al.*[28]), because alkaline bismuth tartrate treatment enhances the diameter of these particles to about twice its original value due to staining of the apoprotein shell. Ferritin is only occasionally apposed close to the lysosomal membrane as can be seen in Fig. 5.

These particles could be clearly differentiated from colloidal iron staining (Figs 6–10). (1) The stain granules were mostly coarser and irregular in appearance. (2) Their contrast was not enhanced by additional section staining. (3) They were predominantly associated with the inside of the bounding membrane or with membrane fragments occurring within lysosomes (Figs 9, 10). (4) Further proof for the specificity of the colloidal iron staining was provided by neuraminidase digestion, which strongly reduces or eliminates the colloidal iron staining (Fig. 7 *versus* Fig. 8).

Normal rat liver lysosomes as well as Triton WR 1339 filled lysosomes from all phases investigated stained with colloidal iron. In many cases the staining was localized predominantly on the inner side of the bounding membrane (Figs 9, 10). An outside localization of the stain was never seen. Staining throughout the thickness of the membrane was observed sometimes; in these cases, however, membrane tilting within the section could not be ruled out. The lysosomal matrix did not stain. Mitochondria, present in light mitochondrial fractions of normal rat liver as well as in "heavy" Triton WR 1339 filled lysosomal fractions remained definitively unreactive (Fig. 6).

Based on these results the inner side of lysosomal membranes may be regarded to behave like a cationic exchanger. This can be mimicked by the adsorption of β-glucuronidase to a strong cationic exchanger (phosphocellulose). Lysosomal contents were prepared, adjusted to the desired pH with acetate buffer (final concentration 0.012 M) and incubated with phosphocellulose (1 mg per assay, 5 min, 22 °C). The nonsedimentable activity of β-glucuronidase was determined after centrifugation

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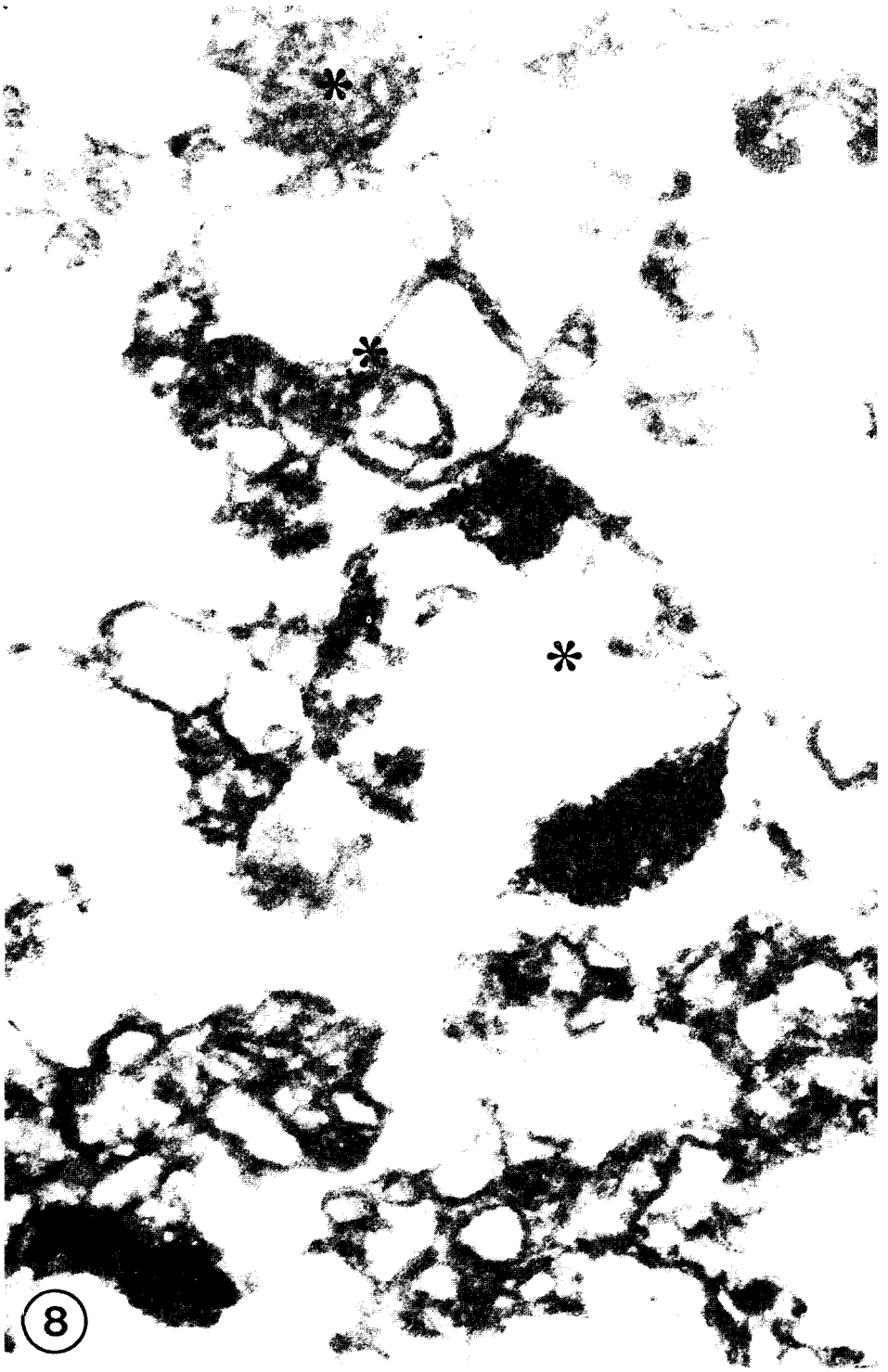
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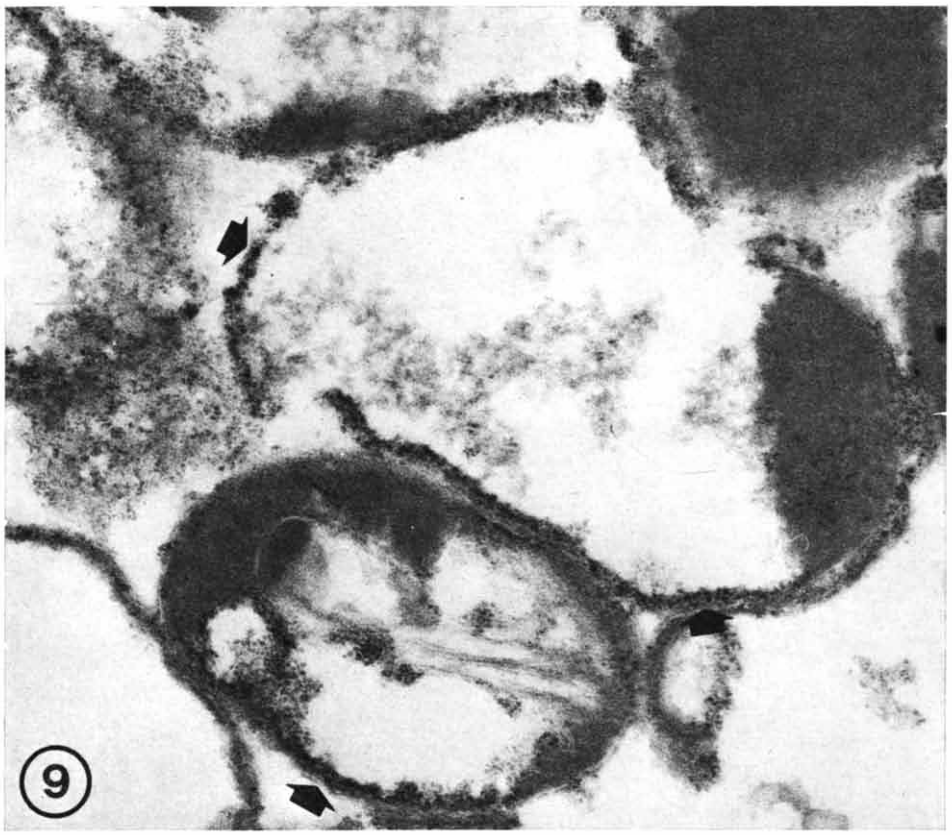
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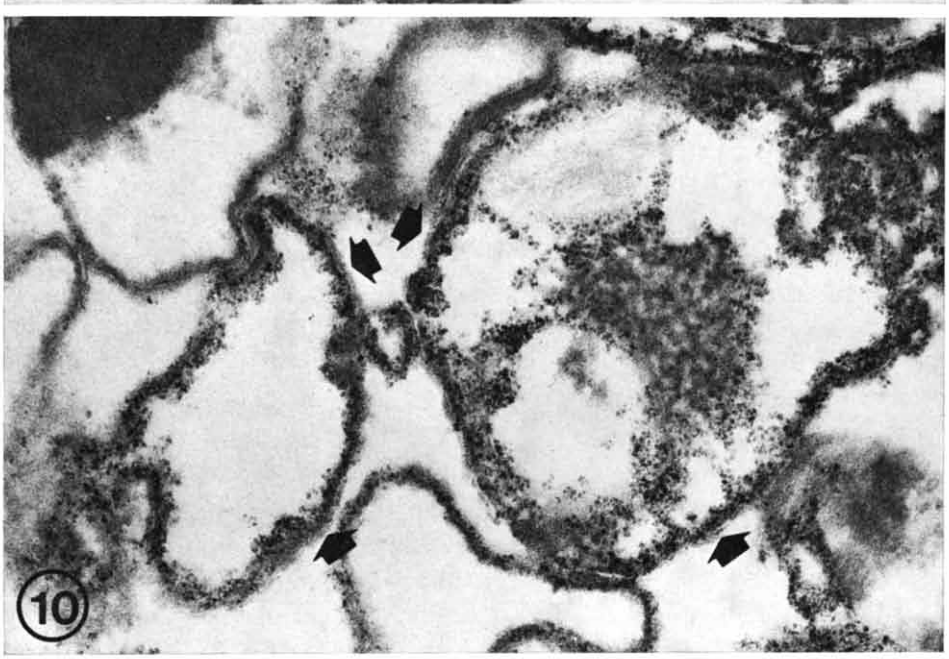
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of the ion exchanger. Fig. 11 a shows the binding curve of β-glucuronidase being pH dependent in a similar manner as seen in Fig. 2 which was obtained with lysosomal membranes. Fig. 11 b demonstrates the influence of the ionic strength on the binding at pH 4 to the ion exchanger indicating again the similarity of this model system with the lysosomal membrane.

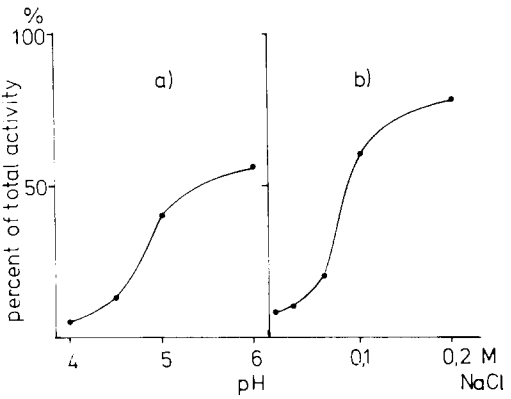

Fig. 11. Influence of pH and ionic strength on the binding of β-glucuronidase to phosphocellulose. (a) Determination of the nonsedimentable activity of β-glucuronidase after incubation of lysosomal contents (0.3 mg protein, 0.037 M acetate buffer of desired pH, 5 min, 22 °C) with 1 mg of phosphocellulose. (b) Determination of the nonsedimentable activity of β-glucuronidase after incubation of lysosomal contents (conditions as under a) at pH 4 with 1 mg of phosphocellulose with increasing NaCl concentrations.

DISCUSSION

The pH-dependent, unspecific and reversible binding of proteins with an isoelectric point around pH 5 to purified lysosomal membranes demonstrates the presence of acidic groups on this type of membranes. Because extensive neuraminidase

Fig. 5. Electron micrograph of Triton WR 1339 filled lysosomes (control). Conventional fixation with glutaraldehyde and osmium tetroxide followed by section staining with magnesium uranyl acetate and lead citrate. No colloidal iron staining. Ferritin granules (arrows) occur mainly in electron dense zones of the matrix but are occasionally associated with the lysosomal membrane. (50000 ×).

Fig. 6. Light mitochondrial fraction of normal rat liver stained with colloidal iron. Glutaraldehyde fixation, section contrasted with hot uranyl acetate in ethanol. Deposits of colloidal iron occur exclusively within lysosomes mainly along the inside of their bounding membrane (arrows). Mitochondria remain unlabelled. (64000 ×).

Fig. 7. Triton WR 1339 filled lysosomes. Preparation as in Fig. 6. Numerous Triton WR 1339 filled lysosomes contain colloidal iron stain on the inside of the bounding membrane. (50000 ×).

Fig. 8. Fragments of Triton WR 1339 filled lysosomes after digestion with neuraminidase. Colloidal iron staining, control to Figs 6, 7, 9 and 10. Exposure to colloidal iron stain remains ineffective with lysosomal fragments (asterisks). (64000 ×).

Figs. 9 and 10. High power electron micrographs of Triton WR 1339 filled lysosomes. Fig. 9. Isolated 24 h after Triton injection. Fig. 10. Isolated 72 h after Triton injection. Preparation as in Fig. 6. The colloidal iron stain is located predominantly on the inner side of the bounding membrane and also along membrane fragments within the interior of Triton filled lysosomes. (94000 ×).

treatment of these membranes is the only means found to influence this binding behaviour, sialic acid is the most probable candidate for these groups. Colloidal iron staining of these groups could be unambiguously distinguished from ferritin particles which are known to occur in normal (Novikoff[27]) and in Triton WR 1339 filled rat liver lysosomes (Wattiaux *et al.*[12]). Inhibition of the staining reaction by neuraminidase pretreatment and lack of staining on contaminating mitochondria support the specificity of the reaction (Figs 6–8).

As to the significance of these results two considerations should be discussed. Firstly the inside localization of sialic acid in secondary lysosomes could arise from the interiorization of the plasma membrane during the pinocytotic process. Combining the spray-freeze etching technique[30] with high resolution shadow-casting[31] as well as by gel chromatography it was found that Triton WR 1339 contains a polymer with 85 Å diameter and with a molecular weight of approximately 100000 which can be taken up by pinocytosis only[10,11]. Benedetti and Emmelot[24] showed the outside localization of sialic acid on rat liver plasma membranes. However, no pH dependent binding of lysosomal enzymes to isolated plasma membranes could be observed.

Secondly an intralysosomal accumulation of nondiffusible anions could be of biological importance in building up a Donnan equilibrium which leads to intralysosomal accumulation of cations such as K^+ and H^+. This would provide a simple means to maintain an acid milieu within lysosomes as already mentioned by Coffey and de Duve[6]. An acid milieu is a prerequisite for the catalytic activity of most of the lysosomal enzymes. Since interiorization of sialic acid occurs during endocytosis, the acid milieu would arise at the moment of fusion between heterophagosomes (whose bounding membrane is derived from the plasma membrane) and primary lysosomes. In this regard pinocytosis could fulfill two tasks, the transport of exogenous material as well as the activation of lysosomal enzymes by the generation of an acid milieu within lysosomes. Such temporal changes in pH within phagocytic vacuoles of polymorphonuclear leucocytes have been observed recently by Jensen and Bainton[29]. These authors found a change of colour in yeast cells stained with indicator dyes within minutes after interiorization indicating a drop of pH down to pH 3.5–4.5.

Auxiliary mechanisms to set up such strong pH gradients must be taken into account. The K^+ influx accompanying the proton influx down to a pH of about 4 would enormously increase the osmotic pressure. Either a selective proton permeability or an energy dependent K^+/H^+ exchange as postulated by Mego *et al.*[9] should be present in the lysosomal membrane.

Finally, it should be emphasized that lysosomes filled with Triton WR 1339 represent a special type (telolysosomes)[10,11] among the various lysosomal classes. Similar studies with other types of pure lysosomal fractions are needed to draw more general conclusions on the importance of the results presented in this study.

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